Investigations on *Piper betle* grown in Sri Lanka


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Abstract

*Piper betle* is an economically important plant cultivated in Sri Lanka. Although more than 12 cultivars of betel are reported in Sri Lanka, very few scientific investigations have been carried out on them. Studies on the chemical constituents indicated that safrole is the major constituent, followed by chavibitol acetate, in the essential oil of common betel leaves of Sri Lanka. Investigations on the bioactivities of *P. betle* revealed the presence of antimicrobial, insecticidal, antioxidant, antinociceptive, antidiabetic and gastroprotective activities. In addition, *P. betle* was found to be safe in terms of hepatotoxicity, renotoxicity, hematotoxicity, gross morphology, weights of organs, stress or aversive behaviors in rats. The above findings indicate the vast potential of *P. betle* yet to be harnessed for the benefit of mankind and the betel industry of Sri Lanka.

Key words: Anatomy, bioactivities, chemical constituents, morphology, *Piper betle*, safety profile

Introduction

*Piper betle* Linn. (Sinhala name: Bulath, English name: Betel) belongs to the genus *Piper* of the plant family Piperales.[1] Over 700 species of plants belonging to the genus *Piper* are distributed in both hemispheres. Of these, about 30 species have been reported from India.[2] In Sri Lanka, 18 species of genus *Piper* are found; and out of them, three are endemic.[3]

Betel leaves have been traditionally used for chewing purposes along with other condiments. Colombo, Gampaha, Kalutara, Kurunegala, Kegalle, Ratnapura, Matale and Galle are the main betel-cultivating districts in the country. In addition to a wide and well spread domestic market, betel has gained a significant position in the export market since 1974. Betel industry, at times, faces severe problems of depressed prices and restricted export market. The main cause for this situation is that Pakistan, our major buyer of betel, has reduced the volume of betel imported from Sri Lanka.[4]

Although betel vine has been cultivated in Sri Lanka for centuries, very few research activities have been carried out on it, except studies on antaphrodisiac activity,[5] antifertility effects on male rats,[6] and antimotility effects on washed human spermatozoa.[7] However, *P. betle* grown in other countries has been shown to possess antimicrobial,[8] gastroprotective,[9] wound healing,[10] hepatoprotective,[11] and antioxidant[12] activities. In order to minimize the negative impact on the betel industry in Sri Lanka, there is a necessity to study important bioactivities of Sri Lanka grown betel leaves and develop value added products based on these activities. This strategy will safeguard both growers and the economy of the country. In order to achieve this, possible bioactivities of betel oil and extracts were investigated using betel leaves from Sri Lanka. In addition, morphological, anatomical and chemical studies were also conducted. These investigations and their results are summarized below.

Classification

Kingdom: Plantae
Order: Piperales
Family: Piperaceae
Genus: *Piper*
Species: *P. betle*

Morphological and anatomical studies of betel
Six cultivars of *P. betle* Linn., namely, Galdalu, Mahamaneru, Kudamaneru, Ratadalu, Nagawalli and Malabulath, were used...
in the present study. Morphological and anatomical features, including parameters such as stomatal index, leaf length-to-width ratio, were similar in Kudamaneru, Mahamaneru, Galdalu, Ratadalu and Nagawalli but were different in the cultivar Malabulath.

CHEMICAL CONSTITUENTS AND PHYSICOCHEMICAL PROPERTIES OF THE ESSENTIAL OIL

Chemical composition
According to the chemical constituents present in the essential oil (EO), cultivars of Galdalu, Mahamaneru, Kudamaneru, Ratadalu and Nagawalli were similar and they were categorized under “common betel.” EOs from the leaves, stalks, stems, fruits and roots of common betel and from the leaves of Malabulath were also analyzed. Major constituents of the EO of common betel were found to be safrole (48.7%) and chavibitol acetate (12.5%). Malabulath does not contain these two compounds. The major compound in Malabulath oil is allylpyrocatechol diacetate (34.0%), which is the third major compound in common betel oil (11.3%). Further, p-cymene, 4-terpineol, safrole, eugenol, β-caryophyllene and chavibitol acetate detected in common betel leaf oil were not detected in Malabulath leaf oil. The Gas Chromatography-Mass Spectroscopy analysis of the EO of different parts of common betel indicated that composition of the stalk oil was different to that of the other parts, as it did not contain detectable amounts of allylpyrocatechol diacetate. The major compound detected in the EO from the leaf, the stem, the stalk and the root was safrole; but in the fruit oil, it was β-phellandrene. This chemical composition of the EO of leaves appears to be closer to that of cultivar Deshawari in India.

The composition of the betel EO changes with the maturity of the leaf. It was observed that the contents of major compounds safrole and chavibitol acetate in the leaf were at the maximum level at the harvesting stage. Moreover, eugenol and β-phellandrene content decreased with maturity, and β-phellandrene content remained constant after maturity. Allyl pyrocatechol diacetate content increased up to the harvesting stage and remained constant thereafter. The study on the variation of the composition of the EO with maturity is useful in deciding the maturity stage at which the leaf has to be collected for applications that depend on specific compounds. Further, it justifies why Ayurvedic physicians mention the maturity of the plant in drug preparations.

Physicochemical properties
The physicochemical properties of the EOs of Kudamaneru, Mahamaneru, Galdalu, Ratadalu and Nagawalli too were similar but were different from those of Malabulath. These studies indicate that physicochemical properties and chemical constituents of the EO of Malabulath are different from those of other cultivars.

ANTIMICROBIAL SCREENING STUDIES

Antibacterial activity
In the present study, the EO from the leaves showed activity against Escherichia coli, Streptococcus pyogenes and Staphylococcus aureus. The Minimum Inhibitory Concentration (MIC) values were 3.12 × 10⁻³, 2.50 × 10⁻¹ and 5.00 × 10⁻¹ µg/mL, respectively. The ethanol extract showed activity against Streptococcus pyogenes, Escherichia coli and Staphylococcus aureus. The MIC values were 1.25 × 10⁻³, 5.00 × 10⁻¹ and 5.00 × 10⁻¹ µg/mL, respectively.

Antifungal study
This assay revealed that EO of P. betle contained at least three fungicidal compounds, and the ethanol extract contained at least one fungicidal compound active against Cladosporium sp. Further, antifungal activity of betel oil was investigated against Colletotrichium sp., Fusarium oxysporum sp., Corynespora cassioid, Rigidoporous sp. and Phytophthora sp., using the disk method. All fungi species except Phytophthora sp. showed significant growth inhibition in betel oil.

INSECTICIDAL ACTIVITIES

Mosquito larvicidal assay
Late 3rd instar larvae of Aedes aegypti were introduced into P. betle EO at 500, 100, 50, 25, 12.5, 6.25 ppm concentrations, and mortalities were recorded after 1 hour and 24 hours. Mortalities of 43% and 100% were observed for 100 and 500 ppm concentrations, respectively, within 1 hour. Compared to the control, significant mortality was observed even at lower concentrations, 25 and 50 ppm, after 24 hours.

Bioassay for housefly (Musca domestica)
In this assay, betel EO (120 µg/cm² in ethanol) showed 100% knock down effect and mortality against Musca domestica.

Bioassay for rice weevil (Sitophilus oryzae)
Betal EO at 1%, 0.8% and 0.5% concentrations was used in this study. Mortality rate of 100% was observed in 1% betel oil solution within ½ hours.

Bioassay for Chrysomya megacephala larvae
Betal EO solutions ranging in concentration from 1% to 4% were prepared using 1% Tween 80, sodium lauryl sulfate (0.05 g per 100 mL, as a stabilizer) and methyl paraben (0.01 g per 100 mL, as a preservative). The 4% and 3% preparations of the oil of betel were effective in killing 100% of the larvae of C. megacephala within 3½ hours, while betel oil at 2% concentration killed 97% of C. megacephala larvae within 4 hours. The positive control, mineral turpentine, also killed the larvae within 4 hours. This shows that betel oil is effective in the treatment of wound myiasis.

Bioassay for Chrysomya bezziana larvae
A study was conducted to evaluate the efficacy of betel EO against the larvae of Chrysomya bezziana in vitro. With 4% betel...
oil, all 1st instar larvae were killed within 2 hours, and the 2nd instar larvae were killed within 4 hours. The positive control (Asuntol) showed no mortality until 4 hours, but all larvae were weak from first 30 minutes. In the negative control, larvae were mobile and active. Betel oil at 3% killed all the 1st instar larvae within 150 minutes; and 74% of the 2nd instar larvae, within 4 hours. These results indicate that betel oil from Sri Lanka is an effective larvicide for C. bezziana 1st and 2nd instar larvae in vitro.

**ANTIOXIDANT ACTIVITY**

The extracts obtained from the leaves of *P. betle* had profound antioxidant activity as judged by Thiobarbituric Acid Reactive Substances (TBARS) and 2, 2 – diphenyl – 1 – picrylhydrazyl (DPPH) scavenging assays.[14,17] The scavenging effects of *P. betle* extracts on DPPH radicals increased in the following order: Cold Ethanolic Extract (CEE) > EO > HWE. Further, free radical scavenging effect of CEE was higher than that of synthetic antioxidant Butylated Hydroxy Toluene (BHT). Employing the DPPH assay, Indian researchers[18] have investigated the antioxidant activities of three betel cultivars (Kauri, Ghanganete and Bagerhati) grown in India. The antioxidant activities of the three cultivars were in the order of Kauri > Ghanganete > Bagerhati, but the free radical scavenging ability of commercial betel from Sri Lanka is higher than that of the reported Indian cultivars.

In TBARS assay, the antioxidant potential of CEE was the best among the *P. betle* extracts tested, and this effect was significantly higher than that of BHT and green tea, respectively. Compared to EO and HWE, the degree of delaying the lipid peroxidation was significantly lower in BHT and in green tea. Safrole is the major constituent in the EO of Sri Lankan commercial betel leaves. However, compared to the EO, the antioxidant activity of safrole was significantly low. This suggests that antioxidant potential of EO is not only due to safrole but possibly due to synergetic effect of all volatile constituents.

Interestingly, the antioxidant properties of *P. betle* extracts, including CEE, EO and HWE, remained unaltered for a period of 12 months at room temperature (as evaluated by DPPH assay). This supports the potential use of the betel extracts as a natural antioxidant in food industry. However, when *P. betle* extracts were exposed to elevated temperature (200°C), the antioxidant property was significantly reduced (CEE and EO by 4 fold; HWE by 3 fold). Similar results were also evident with the synthetic antioxidant BHT (by 4 fold). Even after exposure to the elevated temperature, the antioxidant potential of CEE was higher than that of BHT.

In an attempt to introduce betel as a natural antioxidant, the CEE was incorporated into fats (cake margarine), oils (coconut and palm oil) separately and its rancidity determined in terms of peroxide value (PV). The results showed that PVs were significantly lower in CEE-treated samples than in BHT-treated samples.

**ANTIDIABETIC ACTIVITY**

Overall results show that both HWE and CEE of *P. betle* leaves from Sri Lanka possess marked hypoglycemic activity (when tested in fasted normoglycemic rats) and antihyperglycemic activity (judged by improvement in the results of glucose tolerance test and by lowering of the blood glucose levels in rats with streptozotocin (STZ)-induced diabetes).[19] The hypoglycemic effect of *P. betle* extracts (100, 200, 300 mg/kg) on fasted normoglycemic rats was dose dependent and lasted up to 4 hours, except that of the lowest dose of HWE. Further, hypoglycemic potential of HWE and CEE, respectively, was comparable to that of tolbutamide, the reference hypoglycemic drug of the sulphonylurea type.

In glucose tolerance test, HWE, CEE and tolbutamide lowered the external glucose level in a similar manner. Further, HWE significantly reduced the blood glucose level of rats with STZ-induced diabetes treated with a dose (50 mg/kg) which is known to irreversibly damage the insulin-secreting β cells of the pancreas. This suggests that an intact endocrine pancreas and insulin are not essential for antidiabetic activity of *P. betle* extracts. This ability of lowering the blood glucose levels of rats with STZ-induced diabetes also suggests that *P. betle* extracts have insulinomimetic activity. It is possible that HWE may act as an insulin secretagogue and/or sensitize insulin receptors, as proposed for some plant extracts. HWE failed to significantly inhibit glucose absorption from the lumen of the intestine. However, HWE provoked accumulation of glycogen in the liver and the skeletal muscle. This is another peripheral mechanism through which HWE exhibits its antidiabetic activity. This increased glycogenesis may result from enhanced glucose uptake from liver and skeletal muscle by sensitization of insulin receptors and/or inducing the activity of enzymes involved in glycogen synthesis.

**GASTROPROTECTIVE ACTIVITY**

A study to evaluate the gastroprotective activity of HWE and CEE of *P. betle* leaves was carried out.[19] Three doses (200, 300 and 500 mg/kg) of both the extracts were evaluated for gastroprotective activity against ethanol-induced gastric ulcers in rats. Oral administration of HWE and CEE provided marked dose-dependent and significant protection against gastric damage caused by absolute ethanol. The gastroprotective effect of HWE was comparable to that of CEE. Further, the gastroprotective activities of the highest dose of both extracts were significantly greater than the gastroprotective activity of misoprostol, the reference drug. A similar study conducted using Indian betel cultivars has been reported.[19] In this experiment, treatment with ethanol extract of betel leaves at a dose of 150 mg/kg for 10 consecutive days produced significant healing effect in rats with ulcers induced by nonsteroidal anti-inflammatory drugs (NSAIDs).

The HWE significantly increased the mucus content (by 49%)
adhering to the wall of the gastric mucosa. Mucus layer is considered to be important in the mucosal defense against endogenous aggressors, e.g., acids, and also as an agent in facilitating its repair. It is generally believed that enhanced acid secretion is the most important factor for the induction of gastric lesions. In this study, the highest dose of HWE did not cause significant inhibition in acidity (both total and free) or pH of gastric fluid. Therefore, the gastroprotective effect of *P. betle* was not mediated via inhibition of acid secretion in the gastric mucosa but by increasing its mucus content.

ANTINOCICEPTIVE ACTIVITY

The CEE and HWE of betel leaves have antinociceptive activity, as evaluated in the hot plate test and in the tail flick test in rats. This indicates centrally mediated antinociceptive activity of the plant extracts against acute pain.[23] The antinociceptive activity of extracts (100, 200, 300 mg/kg) was genuine, as there was no change in the retain retention times in the Bar and Bridge tests as compared to controls. Both 200 and 300 mg/kg doses of *P. betle* extracts markedly reduced the licking time in early and late phases of the formalin test in a bell-shaped dose-response curve. In the formalin test, the pain in the early phase is caused due to the direct stimulation of the sensory nerve fibers by formalin, while the pain in the late phase is due to the inflammatory mediators, like histamine, prostaglandin, serotonin and bradykinin. It is reported that NSAIDs reduce both phases of the formalin test. The betel extracts too induced interruptions in both phases of this test, suggesting possible impairments of sensory transmission and release of inflammatory mediators. The highest antinociceptive activity was evident with 200 mg/kg dose of both HWE and CEE. As antinociceptive activity of CEE was higher than that of HWE, CEE was used to investigate its antinociceptive mechanism.

Some sedatives possess antinociceptive activity. However, the antinociceptive action of CEE is unlikely to be mediated via sedation, as none of the parameters monitored in the Rat hole board technique were changed. Antinociception can be induced via dopaminergic mechanisms, but such a mode of action is unlikely in this study as metoclopramide, a dopamine receptor antagonist, failed to block antinociception induced by the extract. The opioid receptor, antagonist naloxone, blocked the antinociception induced by CEE, suggesting that the antinociception was mediated through opioid mechanisms.

SAFETY PROFILE

There were no treatment-related deaths or morbidity with CEE (1,500 mg/kg/d) and HWE (1,500 mg/kg/d) even after following sub-chronic (14 days) and chronic (42 days) oral treatment in rats.[24] Further, CEE and HWE treated rats showed normal food intake, water intake, and their percentage weight gain significantly reduced. The consistencies of feces and color of urine in CEE and HWE treated rats were essentially similar to those of the controls. Furthermore, neither extract of *P. betle* induced any overt signs of toxicity (salivation, diarrhea, lacrimation, tremors, ataxia, yellowing of hair, loss of hair, postural abnormalities or behavioral changes), stress (fur erection or exophthalmia) or aversive behaviors (biting paw and penis, intense grooming behavior, scratching behavior, licking at tail or vocalization).

Neither extract significantly changed any of the serum parameters [aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine] and hematological [red blood cell (RBC) counts, white blood cell (WBC) counts and hemoglobin (Hb) concentration] parameters investigated. All the tested organs (liver, kidneys, testes, adrenal glands, heart, spleen, vasa deferentia, prostate glands, seminal vesicles together with coagulating glands and caput plus corpus epididymides) appeared normal in all treated rats. There were no significant changes in the organ weights between the treated groups except for the spleen. Compared to the control, in both treated groups significant increase in weight of the spleen was evident (CEE- 217.4%; HWE- 234.8%). Gastric lesions were not observed in any of the treated rats. In conclusion, this study shows that both cold ethanolic and hot water extracts of Sri Lankan betel leaves and leaf stalk were safe following sub-chronic oral administration to rats.

A similar study conducted in India also reported that ethanolic extract of *P. betle* leaf stalks was nontoxic as judged by hematological, biochemical profiles and enzymatic studies.[23]

DEVELOPMENT OF VALUE-ADDED PRODUCTS FROM BETEL

Based on the results of scientific investigations, several value-added products such as betel toothpaste, mouthwash, face cream, shampoos, instant betel quid, betel pellet, antitick lotion, antitick powder and wound healing creams were developed in order to enhance the marketability of betel and improve the prospects of the industry. Clinical trial conducted using the wound healing cream on dermatitis patients revealed that treatment was significantly effective on skin rashes.[25] At present a clinical study is in progress to evaluate the antidiabetic activity of spray-dried powder of betel hot water extract.

CONCLUSIONS

*P. betle* is a common plant in Sri Lanka, and it can be easily cultivated in any part of the country. This scientific study revealed for the first time the chemical constituents and multifaceted activities of betel cultivated in Sri Lanka. The *Chrysomya megacephala* and *C. bezziana* larvicidal activities of betel as well as its antidiabetic and antinociceptive activities have been reported for the first time, herein. The fact that betel leaves have multiple activities such as antimicrobial, insecticidal, antioxidant, antinociceptive, gastroprotective and antidiabetic, as revealed in this study, indicates that *P. betle* is a good candidate...
for future herbal drug preparations and development. This hitherto untapped vast potential of betel grown in Sri Lanka, if properly harnessed, will safeguard the betel industry of Sri Lanka, enhance the livelihood of a large number of villagers depending on betel industry and introduce novel herbal products and drug preparations into the market.

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